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PRINCIPAL INVESTIGATOR: William H. McBride

CONTRACTING ORGANIZATION: University of California
                          Los Angeles, CA 90024

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14. ABSTRACT
Radiation therapy (RT) is the front line treatment for prostate cancer in the early stages but is relatively ineffective against large tumor volumes and it is difficult to use it against micrometastatic disease. Immunotherapy (IT) has become popular as an alternative treatment since the discovery of prostate tumor-associated antigens (TAA) and of corresponding tumor-specific T cells in prostate cancer patients. However, IT is not a very effective modality on its own due to multiple tumor escape mechanisms and probably would benefit from combination with other therapies, such as RT. At least in theory, a potential advantage is that radiation affects the immune system by upregulating MHC class I and co-stimulatory molecules, which could promote T cell filtration into tumors and T cell activation. On the other hand, we recently showed that radiation affects proteasome function, which could affect antigen processing, and appears to have other effects on DC antigen presentation. In the first year of this study, we have been constructing cell lines to examine this in prostate cancer. We have also examined the effects of radiation on DC processing PSA either endogenously or exogenously as well as on proteasome and immunoproteasome function in DCs. The final goal of the proposal is to determine if radiation affects the hierarchy of antigenic peptide presented by DCs and tumor cells and to devise better strategies in combination treatments of RT and IT.

15. SUBJECT TERMS
Radiation, Dendritic Cells, PSA, Proteasome

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Introduction

Radiation therapy (RT) is a very effective treatment for early stage cancer but not for large tumors or for distant micrometastatic disease. Recently, immunotherapy (IT) has gained in popularity by finding some patients have specific T cell response to prostate tumor-associated antigens such as prostate specific antigen (PSA) and this can be boosted by vaccination. On the other hand, IT is not a very effective clinical option in prostate cancer and combining it with RT is to be an attractive concept as it might improve the therapeutic effects of both treatments. There are studies showing that radiation upregulates the expression of MHC class I and immune co-stimulatory molecules, some of which we have performed. We proposed that radiation generates these "danger" signals and modulates tumor microenvironment. Our previous study has shown that radiation inhibits dendritic cell (DC) endogenous processing of MART-1 antigen but enhances exogenous MART-1 peptide presentation. We hypothesized that the inhibitory effect of radiation is due to inhibition of 26S proteasome function, which is critical for the generation of immunopeptides. This large multi-subunit protein is composed of core 20S and 19S regulator structures. IFN-gamma treatment of cells causes replacement of constitutive 20S enzymes subunits with LMP7, LMP2, and MECL-1 and the 19S regulator with an 11S complex, forming an immunoproteasome that favors cleavage of proteins into peptides better able to bind MHC class I molecules. However, most cells do not express immunoproteasome with the notable exception of DC. If tumors express different epitopes than the DC the responses that are generated are unlikely to be effective. In this proposal, we will examine how RT affects the immunological landscape of anti-tumor immunity by altering antigenic epitope presentation by DC and tumor.

Body

This has been a year spent in preparing the ground for future experiments by developing cell lines and reagents as well as refining our experiment approaches. Our hypothesis is that radiation affects proteasome function and modifies peptides presented by DC and tumor. Our previous study on MART-1 system has shown that irradiated DC presented exogenous peptide more efficiently and antigen that was processed endogenously less efficiently. The hypothesis therefore was that irradiation skews the immune system. One obvious question was whether or not this applied to prostate tumor antigens. To examine whole PSA protein processing and presentation, we had to develop a humanized mouse model. We placed PSA within an adenoviral delivery vehicle to express it within DCs. As can be seen in Fig. 1, DCs transduced with AdVPSA and used to immunize mice within the context of HLA-A2/Kb were compromised following irradiation. IFN-gamma expression as judged in an ELISPOT assay was decreased in the irradiated-DC injected group. To determine if the exogenous pathway of presentation was affected, we used the immunodominant peptide PSA-3 peptide to pulse irradiated and non-irradiated DCs. In contrast to the endogenous pathway, exogenous peptide-pulsed DC showed enhanced IFN-gamma and IL-4 expression following 10 Gy radiation treatment (Fig.2). This clearly indicates that it is not a question of cell viability, but an alteration in DC function following irradiation.
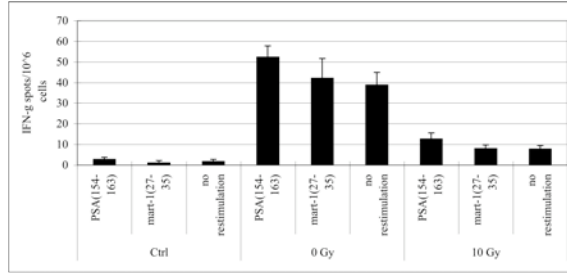
Fig. 1: Radiation effects on PSA processing by humanized HLA-A2/Kb DCs. HLA-A2.1 mice were injected with DCs treated with or without 10 Gy and with AdVPSA. Spleens were harvested 10-14 days after DC immunization and restimulated with either PSA-3 or non-specific peptide MART-1 (27-35) or no stimulation. The production of IFN-gamma was assessed by ELISPOT.
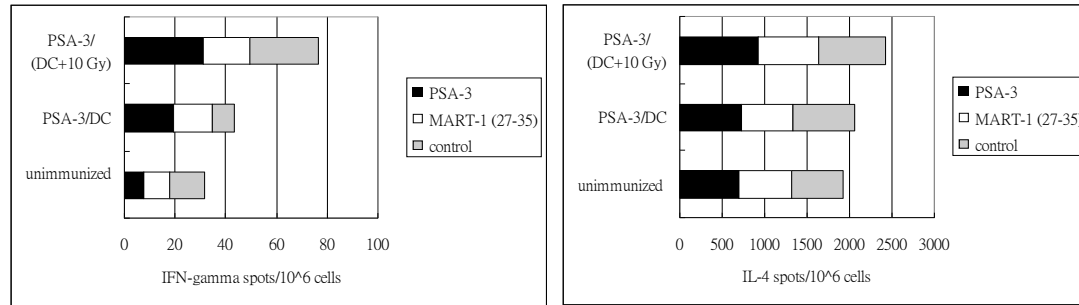

Fig. 2. Radiation effects on endogenous antigen processing by DCs. HLA-A2.1 mice were injected with DCs treated with or without 10 Gy and pulsed with PSA-3 peptide. Spleens were harvested 10-14 days after DC immunization and restimulated with either PSA-3 or non-specific peptide MART-1 (27-35) or no stimulation. The production of IFN-gamma and IL-4 were assessed by ELISPOT.

We have been involved in developing PSA-expressing murine tumor lines to determine if tumor rejection is affected, but while doing this, we examined the effect of RT on the stability of MHC class I molecules in a classic model using T2 cells, the hypothesis being that radiation stabilizes MHC class I expression on cells. T2 cells are TAP-deficient human lymphoblast cells with empty MHC class I complexes on their surface that are inherently unstable. Pulsing these cells with peptides stabilizes MHC class I, which can be measured by flow cytometry. Irradiation of 2 Gy and 10 Gy appears to increase the levels of stable MHC I complexes over a 24 h period suggesting a stabilization effect by radiation (Fig. 3). This indicates that the radiation-induced changes are membrane-associated. We then further tested the radiation effects on lipids by examining the lipid rafts. Lipid rafts are sphingolipid- and cholesterol-rich membrane microdomains that sequester GPI-linked protein and other signaling proteins and receptors. Bone marrow-derived DCs (BMDCs) were treated with 10 Gy radiation and/or methyl-beta-cyclodextrin (MβCD). Radiation enhanced the drifting of lipid rafts as shown in Fig. 4: green fluorescence spots were accumulated in 10 Gy-treated group compared to untreated DCs and MβCD-treated cells. We will continue the study as stated in the proposal to explore the mechanism of radiation-induced effects on stability and turnover of MHC class I molecules in PSA-TRAMP C1, -C2 and -DC 2.4.
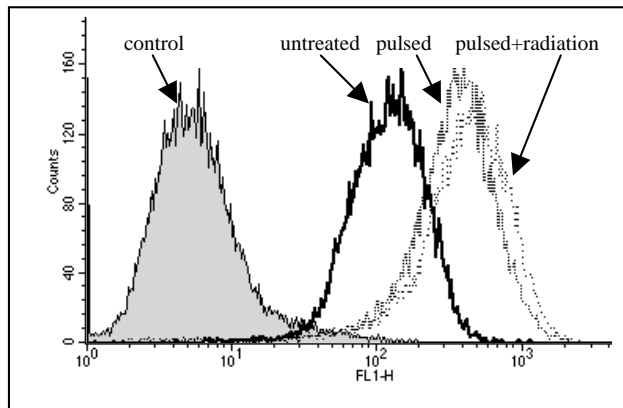
Fig. 3. Flow cytometric analysis of MHC class I on T2 cells. T2 cells were treated with 2 Gy and 10 Gy and tested for MHC class I expression 24 h after treatment. It seems that radiation increases the levels of stable MHC I complexes over a 24 h period, an effect that is abolished in the presence of the Golgi inhibitor brefeldin A.
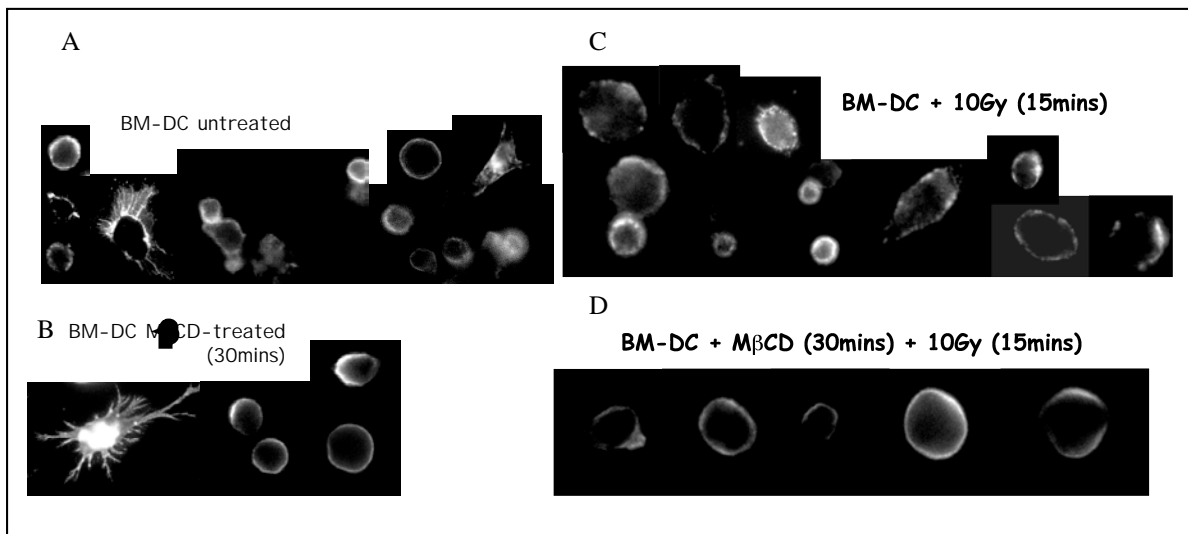
Fig.4. Radiation enhances lipid rafts drifting in bone-marrow derived DCs. DCs were treated with cholesterol depleting drug methyl-beta-cyclodextrin (MβCD) for 30 min and/or 10 Gy. Fifteen minutes after irradiation, DCs were stained with green fluorescent Alexa Fluor dye and viewed under fluorescence microscope. (A) untreated, (B) MbCD treatment, (C) 10 Gy, (D) MbCD +10 Gy treatments.

As proposed in aim 2, we have examined the radiation effects on proteasome function of the parental cells that we are inserting PSA in. As expected, radiation treatment of TRAMP C1 and BMDC decreased proteasome chymotrypsin-like activity (Fig. 5).

Fig. 5. Proteasome chymotrypsin-like activity of (A) TRAMP C1 and (B) BMDC cells were inhibited ~40% by 10 Gy irradiation. Fluorescence was measured from substrate cleaved by the crude cell extracts of cells treated with 10 Gy irradiation. Relative fluorescent units represe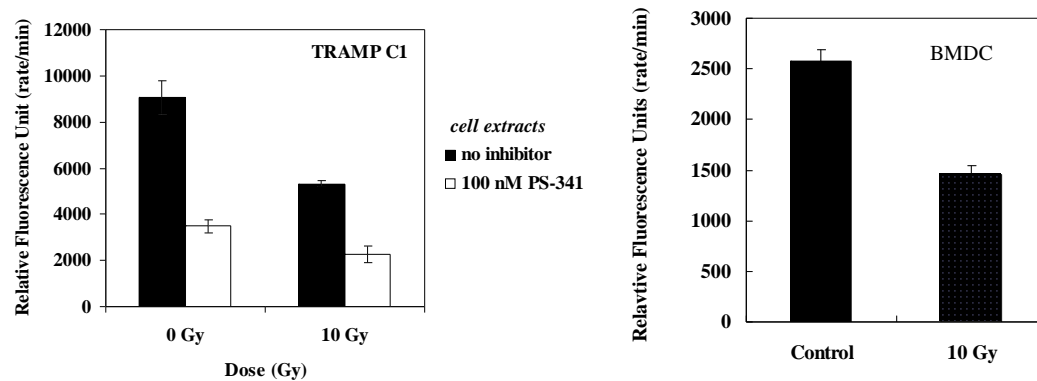nt activity per 10 μg total protein. Crude cell extracts were incubated with 100 nM PS-341 (Velcade) for 10 minutes prior to assay in order to assess the inhibited proteasome activity in A.

The ratio of constitutive proteasomes to immunoproteasomes could be altered by radiation and a series of experiments were designed to test whether this was the case. Our previous study has shown that irradiation does not affect proteasome function in T2 lymphoma cells. Since these cells lack a large segment of genes encoding HLA class II, LMP7 and LMP2, and TAP1 and TAP2 on chromosome 6, they are generally considered to lack immunoproteasomes since LMP7 is required for LMP2 and MECL-1 assembly. To test this further, we used immunoproteasome-deficient LMP7, LMP2 and MECL-1 knockout mice. We anticipated that DCs generated from these knockout mice would behave the same since these three IFN-gamma-subunits are co-regulated and proteasome function in DC from LMP7 and LMP2 knockout mice will be less responsive to radiation, since T2 cells were not sensitive to radiation. Indeed, irradiation (2 Gy or 10 Gy) of DCs from LMP7, LMP2 and MECL-1 knockout mice had less effect as judged by chymotrypsin-like activities of proteasome extracts with or without 48-hr 500 U/ml IFN-gamma stimulation (Fig. 6) as did treatement with IFN-gamma. Similar experiments will be performed later this year to confirm the findings using PSA-expressing cell lines.
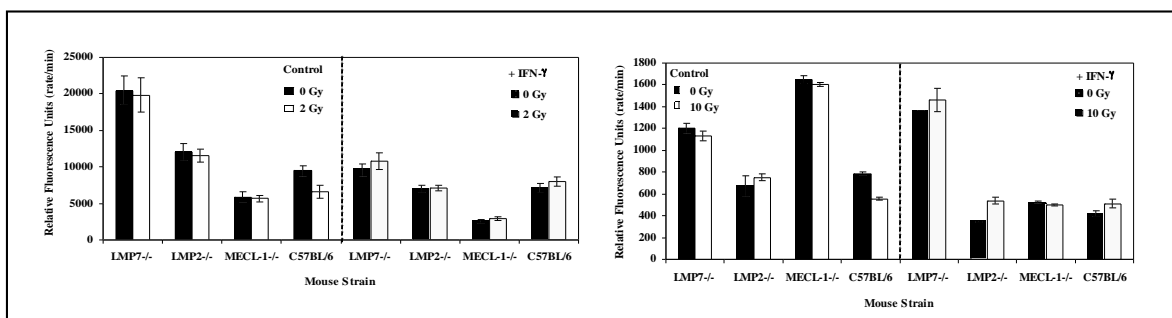
Fig. 6. Irradiation had no effects on IFN-γ treated DC proteasome activity. DCs from LMP7, LMP2 and MECL-1 knockout (LMP7-/-; LMP2-/-; MECL-1-/-) mice and C57BL/6 mice were treated with or without 500 U/ml IFN-gamma for 48 hours prior to (A) 2 Gy or (B) 10 Gy irradiation. Proteasomes were then extracted and chymotrysin-like activity was measured using fluorogenic substrate. Relative fluorescence units represent activity per 10 μg of total protein

Key Research Accomplishment

1. Development of PSA-TRAMP C1, PSA-TRAMP C2 – 90% completed.
2. Development of PSA-DC 2.4 – 80% completed.
3. Study of the radiation effect on stability of MHC class I molecules -60% completed.
4. Study of radiation effects on proteasome and immunoproteasome function – 50% completed.

Reportable Outcomes

Kim, K, James M. Brush, Philip A. Watson, Nicholas A. Cacalano, Keisuke S. Iwamoto, and William H. McBride. EGFRvIII Expression in U87 Glioblastoma Cells Alters their Proteasome Composition, Function, and Response to Irradiation. <u>Molecular Cancer Research</u>. in press.

1/07
McBride, W. H., Invited speaker: "Radiation affects the rate of protein degradation." University of Texas, Division of Radiation Biology/Department of Radiation Oncology Seminar Series.

5/07
McBride, W. H., "The proteasome in radiation response." 10th International Wolfsberg Meeting, Ermatingen, Switzerland.

7/7-7/12/07
McBride, W. H., Symposium speaker and abstract presentation: "Radiation affects on the composition and function of immune cells within the tumor microenvironment." 13th International Congress of Radiation Research, San Francisco, CA.

Conclusions

      This has been a year preparing cell lines and developing reagents and investigating the effect of radiation on DC function, stability of MHC class I molecules and proteasome function. We examined the effects of radiation on PSA presentation by DCs. Radiation inhibited endogenous PSA processing while enhancing exogenous PSA peptide presentation. The observations that radiation stabilized peptide-pulsed MHC class I molecules and drifted lipid rafts on DC surface support the finding that the immune responses were enhanced in irradiated PSA-3 peptide-pulsed DCs. We have also investigated the effects of radiation on different proteasome composition in DCs. IFN-gamma treatment on DCs showed less inhibitory effect on proteasome function following RT. In this year, we will continue our study using PSA-expressing cell lines to confirm what we have proposed in the statement of work.

Appendices: one accepted manuscript.

EGFRvIII Expression in U87 Glioblastoma Cells Alters their Proteasome Composition, Function, and Response to Irradiation.

Kwanghee Kim[1], James M. Brush[1], Philip A. Watson[2], Nicholas A. Cacalano[1], Keisuke S. Iwamoto[1], and William H. McBride[1]

[1] *Department of Radiation Oncology, Experimental Division, David Geffen School of Medicine at UCLA, University of California Los Angeles, Los Angeles, California and [2] Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, New York.*

Request for reprints: William H. McBride

Department of Radiation Oncology,

Roy E. Coats Research Laboratories

Room B3-109

David Geffen School of Medicine at UCLA

10833 Le Conte Ave., Los Angeles, CA 90095-1714

Phone: 310-794-7051

Fax: 310-206-1249

E-mail: wmcbride@mednet.ucla.edu

A running title: ionizing radiation induced changes in proteasome expression

Abstract

Little is known about the factors that influence the proteasome structures in cells and their activity, although this could be highly relevant to cancer therapy. We have previously shown that, within minutes, irradiation inhibits substrate degradation by the 26S proteasome in most cell types. Here we report an exception in U87 glioblastoma cells transduced to express the EGFRvIII mutant (U87EGFRvIII), which does not respond to irradiation with 26S proteasome inhibition. This was assessed using either a fluorogenic substrate or a reporter gene; the ornithine decarboxylase degron fused to ZsGreen (cODCZsGreen), which targets the protein to the 26S proteasome. To elucidate whether this was due to alterations in proteasome composition, we used qRT-PCR to quantify the constitutive (X, Y, Z) and inducible 20S subunits (Lmp7, Lmp2, Mecl1), and 11S (PA28alpha and beta) and 19S components (PSMC1 and PSMD4). U87 and U87EGFRvIII differed significantly in expression of proteasome subunits, and in particular immuno-subunits. Interestingly, 2 Gy irradiation of U87 increased subunit expression levels by 16-324% at 6 hours, with a coincident 30% decrease in levels of the proteasome substrate c-myc, while they changed little in U87EGFRvIII. Responses similar to 2 Gy were seen in U87 treated with a proteasome inhibitor, NPI0052, suggesting that proteasome inhibition induced replacement of subunits independent of the means of inhibition. Our data clearly indicate that the composition and function of the 26S proteasome can be changed by expression of the EGFRvIII. How this relates to the increased radioresistancy associated with this cell line remains to be established.

Introduction

The ubiquitin-proteasome pathway degrades most intracellular proteins. Proteasomes exist in several different forms, each focused on degrading primarily different sets of substrates. The 20S core structure contains three different active sites, called β5 (X), β1 (Y), and β2 (Z), responsible for chymotrypsin-like, trypsin-like, and post-glutamyl peptide hydrolase-like (caspase-like) proteolytic activity, respectively. The 20S core is activated by binding either 19S or 11S regulatory complexes (1, 2). The 26S proteasome, which is composed of a 20S core and 19S regulatory subunits, plays an important role in regulating expression of signaling molecules in an ATP-dependent manner (3-5). The 19S regulatory subunits consist of a base structure containing 6 ATPase subunits and 2 non-ATPase subunits, and a lid with non-ATPase subunits. In contrast, binding of 20S core structure to 11S activator components, PA28α and PA28β, is induced by pro-inflammatory signals such as interferon-γ and TNF-α. These stimuli also replace the enzymatic core subunits, X, Y, or Z, with corresponding immuno-subunits Lmp7, Lmp2, or Mecl1 (6, 7). The main substrates of the 20S-11S complex are partially degraded proteins and peptides and the process is ATP/ubiquitin-independent (8).

Proteasome composition and number and hence the rate and specificity of proteolysis, varies by cell type and pathological condition. Cancer cells generally display higher levels of proteasome activity than normal cells and their increased degradation of regulatory molecules such as p27 and TGF-β have been linked to loss of growth control (9-11). This, and the differential responses cancer cells display to proteasome inhibition, makes the proteasome a valid target for cancer therapy. The proteasome inhibitor, Bortezomib (Velcade), was the first approved for treatment of refractory multiple

myeloma and its success has led to the development of additional proteasome inhibitors (12, 13).

We and others (14-18) have shown that drugs that inhibit proteasome activity, such as Bortezomib, radiosensitize cancer cells. In our case, this was a natural progression from our finding that acute irradiation of cells rapidly inhibits all 3 major enzyme activities of the 26S proteasome, with activity being gradually restored over 24 hours. Irradiation of a wide range of cancer cell lines caused a 25-60% decrease in the rate at which proteasomes cleave fluorogenic substrates (19). This observation was dose-independent with maximum inhibition even at low doses. The inhibition of 26S proteasome function was correlated to increased expression of IkB-α, a substrate of the 26S proteasome at low doses of radiation (19), and a general increase in ubiquitinated proteins (20). Remarkably, the activities of purified proteasome preparations irradiated on ice were also reduced (19, 20), indicating that the proteasome is a direct target of radiation. Further study showed that only 26S, not 20S or 20S +11S, proteasomes are affected by radiation and its inhibition is ATP-dependent, suggesting that ATPase subunits of the 19S cap are probable mediators of the response (20).

Glioblastoma multiforme (GBM) is the most common brain cancer of adults and the median survival time of patients with GBM is 12 months from diagnosis (21). The most commonly altered gene in GBM is EGFR, with overexpression occurring in 40-50% of cases, and nearly 50% of these coexpress mutant EGFRs, mostly the type III EGFR variant (EGFRvIII)(22). The EGFRvIII mutant contains an in-frame deletion of amino acids 6-273 resulting in a ligand-independent, constitutively active oncoprotein. EGFRvIII expression has been linked to poor prognosis (23, 24) and implicated in

modulating radiosensitivity (25). Ionizing radiation in the therapeutic dose range of 1-5 Gy increases tyrosine phosphorylation of EGFR wild type (EGFRwt) and EGFRvIII, activating MAPK and PI3K pathways in CHO cells (26). In addition, Lal et al. reported that transcripts of molecular effectors of tumor invasion such as extracellular matrix components, metalloproteases and a serine protease were up-regulated in an EGFRvIII expressing GBM cell line (27). We also noted that EGFRvIII expression led to radioresistance of U87 GBM cells and asked whether this pathway might also affect proteasome composition and radiation-induced inhibition of proteasome function.

Results

Radiation-induced Proteasome Inhibition is Observed in U87, but not in U87EGFRvIII Cells

First, a clonogenic survival assay was used to determine how chronic EGFR signaling modulates the response of U87 cells to single doses of 0, 2, 4, 6, 8 Gy ionizing radiation (Figure 1A). U87 is a radioresistant cell line, but transduction with EGFRvIII made it even more radioresistant, although this was only evident at higher radiation doses.

We had previously shown that irradiation of a wide range of cell types slows the rate at which fluorogenic substrates are degraded through the proteasome. To determine if EGFRvIII expression alters radiation-induced proteasome inhibition, we compared the U87 and U87EGFRvIII cell lines using the same fluorogenic assay for chymotrypsin-like activity after 10 Gy irradiation using Suc-LLVY-AMC peptide (Figure 1B). A 16% higher (P <0.01) proteasomal activity was observed in extracts from untreated

U87EGFRvIII compared to the untreated U87 cell line. U87EGFRvIII also responded to irradiation differently from U87. Irradiation at 10 Gy inhibited 22% (P <0.001) of the proteasome activity in U87, while irradiation actually increased activity in U87EGFRvIII cells (P <0.001), which is in our experience unique and exceptional. 2Gy irradiation also inhibited the proteasome activity in U87 (23%, P <0.0001, data not shown), but not in U87EGFRvIII (2% inhibition, N.S.).

Irradiation Inhibits 26S Proteasome Activity in U87, but not in U87EGFRvIII Cells

In order to determine if protein degradation by the 26S proteasome was similarly inhibited in living cells by irradiation, a reporter gene assay using the cODCZsGreen fusion system was employed (Figure 2). The murine ODC degron (cODC) targets proteins for degradation specifically through the 26S proteasome (28-30) independent of ubiquitin conjugation. Fusion of cODC to fluorescent proteins produces a reporter gene product that does not accumulate in normal conditions but can be used to assess proteasome inhibition (31). We generated stable clones of U87 and U87EGFRvIII expressing cODCZsGreen fusion protein and examined 12 clones of U87-cODCZsGreen and 16 clones of U87EGFRvIII-cODCZsGreen to account for possible clonal variation. These were irradiated at 10 Gy, harvested after 6 hours and the fluorescence of each clone (paired 0 Gy and 10 Gy) was determined using flow cytometry. The mean fluorescence of each independent clone irradiated at 10 Gy was then normalized to that of the corresponding untreated control (Figure 2A). Consistent with the reduction of proteasome activity in U87 seen in Figure 1, 26S proteasome function was inhibited by irradiation resulting in a fluorescence increase in most of the U87-cODCZsGreen clones

(P <0.005) while the fluorescence of U87EGFRvIII-cODCZsGreen clones was little altered after irradiation (P =0.298). The average increase in the mean fluorescence after irradiation was 29.5% for U87-cODCZsGreen and 1.7% for U87EGFRvIII-cODCZsGreen.

To further support the flow cytometry results and to determine the subcellular localization of the fluorescence, clone 9 of U87-cODCZsGreen was additionally analyzed utilizing confocal microscopy. Cells were plated overnight and fluorescence was detected after cells were irradiated with 2 Gy. Accumulation of ODC in U87-cODCZsGreen was observed as early as 15 minutes after irradiation and became prominent in the perinuclear region by 45 minutes (Figure 2B). Similar spatial accumulation of fluorescence was observed in a different cell line (32). This result demonstrates that irradiation affects degradation of physiological substrates as well as of fluorogenic peptides in U87 cells, but not in U87EGFRvIII cells.

Proteasome Composition Varies between U87 and its Isogenic Derivatives

In order to determine if the differential cellular response to irradiation of U87 and U87EGFRvIII cells could be attributed to differences in proteasome composition, we next measured the proteasome subunit expression of U87 isogenic cell lines. The mRNA expression of 10 proteasome subunits was measured in U87, U87EGFR and U87EGFRvIII cells using qRT-PCR. These include the constitutive subunits (X, Y, Z), their inducible counterparts (Lmp7, Lmp2, Mecl1), the 11S subunits (PA28 alpha and beta), and the 19S components (PSMC1 and PSMD4). The expression of each subunit was normalized to GAPDH. For each cell line, gene expression values are shown relative

to the expression of the constitutive subunit X, which was set equal to one (Figure 3). The U87EGFRvIII cell line displayed a very different expression pattern compared to U87 or U87EGFR, which were similar to each other. Expression differences were most prevalent among the immuno-subunits (Lmp7, Lmp2, Mecl1), which were markedly higher in the parental line and down-regulated in the EGFRvIII cells. 19S regulator and 11S activator levels appeared to be similar in the different isogenic lines.

Irradiation Increased the mRNA Levels for All of the Proteasome Subunits in U87, but little in the Radioresistant U87EGFRvIII

Having determined that the proteasomes of the more radioresistant U87EGFRvIII cell line were significantly different from those of U87 in their subunit composition and in their resistance to radiation-induced inhibition, we then asked whether ionizing radiation changes the rate of synthesis of proteasome mRNA and whether this is altered by constitutively active EGFR signaling. We measured the mRNA expression of 10 different proteasome subunits in U87 and U87EGFRvIII cells 6 and 24 hours after irradiation with 2 Gy by qRT-PCR. GAPDH normalized expression was compared to mRNA expression levels of non-irradiated samples (expression of each subunit at 0 Gy =1). At 6 hours after 2 Gy irradiation, the expression of all proteasome subunits tested in U87 cells was increased by 16-324% (Figure 4A). In particular, all three constitutive-subunits of the 20S core were induced by irradiation (summarized in Figure 5B). Consistent with the increase of proteasome mRNA, levels of the proteasome substrate c-myc were decreased by 30% (data not shown). On the other hand, in U87EGFRvIII cells, the extent of upregulation after 2 Gy irradiation was minimal. At 24 hours after

irradiation, neither cell line showed significant changes in their expression compared to untreated controls (Figure 4B), in keeping with restoration of normal proteasome activity.

The increased protein expression of X subunit (beta 5, chymotrypsin-like activity) was also observed by Western blot in U87 whole cell lysates prepared 6 hours after 2 Gy and 10 Gy, while there was no change in protein expression in U87EGFRvIII (Figure 4C). This was the subunit that changed most by qRT-PCR. Radiation-induced changes in Y and Z subunit proteins, which changed less, were not detectable in either U87 or U87EGFRvIII (data not shown), which may have been due to the relative insensitivity of the method.

A Proteasome Inhibitor Shows a Similar Effect as Irradiation on mRNA Proteasome in U87 Cells

To determine if the radiation-induced induction of proteasome subunits was simply a consequence of proteasome inhibition, or something restricted to irradiation, we used an orally bioactive proteasome inhibitor NPI0052 (12) that binds directly to all 3 protease sites and that has been suggested to be more potent than PS341 (Bortezomib). In our system, NPI0052 inhibited >99% of proteasome activity in both U87 and U87EGFRvIII cell lines when they were treated at 50 nM for 3 hours (data not shown). If proteasome inhibition was required for new gene expression, we anticipated that NPI0052 would induce proteasome mRNA expression in both U87 and U87EGFRvIII cells, while irradiation would only do so in the former. The results are shown in Figure 5A, with the expression of each gene normalized to GAPDH and compared to vehicle controls (0 nM NPI0052 =1). Treatment with NPI0052 induced the expression of almost all the subunits

tested in U87, including both core constitutive as well as immuno-subunits (also summarized in Figure 5B). In U87EGFRvIII cells, NPI0052 induced 6 subunits, including core constitutive, but not immuno-subunits. The ability of NPI0052, but not irradiation, to induce constitutive core subunits may be related to the fact that the drug inactivates these, while irradiation acts on the 19S cap (20). The inability of NPI0052 to induce immuno-proteasome production in U87EGFRvIII cells is consistent with the fact that these subunits are poorly expressed in such cells (Fig 3). When the U87 cell line was treated with NPI0052 followed by irradiation, the radiation did not appreciably augment the changes in subunits brought about by NPI0052 alone (data not shown).

Discussion

The U87EGFRvIII cell line is more radioresistant than the U87 parental line, as shown using a clonogenic survival assay (Figure 1A). Modulation of radiosensitivity by the EGFRvIII mutant has been reported *in vitro* and *in vivo* (26, 33). In *in vitro* studies, the expression of EGFRvIII by either transfection or adenoviral delivery enhanced radioresistancy in CHO and U373 cell lines, respectively. Our laboratory previously observed that the activity of the proteasome varies considerably between cell lines, but is reduced 25-60% by ionizing radiation in all of the tumor cell lines tested (34, 35). In this study, irradiation decreased proteasome activity by 22% in U87. While the extent of the inhibition may seem small, it obviously can affect the expression levels of short-lived proteins, as can be seen by the rapid accumulation of the reporter gene, cODCZsGreen, after 2 Gy irradiation. It has been well-established that this protein is targeted to the 26S

proteasome for degradation without ubiquitination (28-30) and its use allows the 26S proteasome activity to be evaluated without interference from effects of radiation on the ubiquitin system. This also clearly indicates that radiation-induced proteasome inhibition has physiological significance and is likely to be highly relevant to radiation-induced changes in levels of important signaling molecules, such as p53, p21, etc. Interestingly, and uniquely, no inhibition was observed in the U87EGFRvIII cell line after irradiation, and indeed an increase in activity was seen.

In addition to the resistance to radiation cytotoxicity and lack of radiation-induced proteasome inhibition conferred by EGFRvIII expression, chronic EGFR signaling also appears to modulate proteasome composition. The mRNA proteasome subunit profile, and in particular that of the immuno-subunits of the core structure is altered in U87EGFRvIII. The lack of core immuno-subunits in EGFRvIII is of interest because this may represent a mechanism of immune escape that is cohesively regulated with overactivation of the EGFR pathway. The differential display of proteasome subunits seems to be specific for EGFRvIII since EGFRwt (Fig 3) or PTEN (not shown) expression did not significantly alter the proteasomal composition from that of the parent line.

The reasons why cell lines differ so much in proteasome composition and activity is uncertain, but there is increasing evidence that the proteasome system responds rapidly to cell environment and stress challenges, which could have consequences in terms of cancer aggression and response to therapy (36). From our data, one can speculate that chronic EGFR signaling in U87EGFRvIII might change the phosphorylation status of proteasome subunits, which may in turn render human glioma tumor cells resistant to

radiotherapy. Roles for phosphorylation of proteasomes have been suggested by others .

Interferon-γ decreases the level of phosphorylation of proteasome alpha subunits, C8

(alpha7) and C9 (alpha3) (37) and the phosphorylation of C8 by casein kinase 2 was

suggested to increase the stability of the 26S proteasome (38). Satoh et al suggested that

dephosphorylation of proteasome structures elicits the dissociation of the 26S proteasome

to the 20S proteasome and the regulatory complex (39). In addition, phosphorylation of

several ATPase subunits of 26S in human lung cancer cell lines was found by 2D gel

analysis (40). Follow-up experiments to determine the phosphorylation status and the

targeted proteasome subunits of EGFR pathway using proteomics tools and/or small

molecule inhibitors of this pathway would be helpful to understand the mechanism of the

radiation resistance conferred by EGFRvIII. It is worth noting that radiation leads to

rapid phosphorylation of EGFR and EGFRvIII and activation of downstream pathways

(33, 41) that might lead to changes in proteasome phosphorylation status and activity.

Previous work from our laboratory illustrated that irradiation decreases proteasome

function within minutes and that activity recovers over the next 24 hours (20). The

mechanism of recovery is not known, but in this study, 2 Gy irradiation increased the

expression levels for proteasome subunits at 6 hours in U87 cells and by 24 hours,

proteasome subunit mRNAs were restored to pre-radiation levels. In the U87EGFRvIII

cell line, where the decrease in activity was absent, there was little induction of mRNA

for proteasomes following irradiation. This is consistent with increased transcription

being a mechanism for the recovery of proteasome activity after radiation-induced

inhibition. The ability of the proteasome system to respond rapidly to challenge is

consistent with the finding of alteration of proteasome subsets in many microarray

studies, including responses to irradiation (42). Snyder et al. observed that downregulation of the 26S proteasome subunits ATPase 2 and E2A/Rad6 was associated with radiation-induced chromosomal instability. On the other hand, irradiation induced protein expression of 20S proteasome subunits α-1 and α-6 after 12 hours. Irradiation has also been reported to down-regulate PA28-γ in the T-lymphocyte leukemic cell line MOLT-4 (shown by 2D gel electrophoresis)(43). Our data would suggest that any radiation-induced change in proteasome subunits may depend on the cell line and pathways that are activated.

Our findings strongly support the hypothesis that the proteasome structure is a direct target of irradiation and differential expression induced following irradiation might further modify changes in the degradation rate of regulatory proteins as well as the recognition of cancers by the immune system. Further studies using approaches such as electron microscopy would be helpful to address the recovery mechanism of proteasome function since the analysis of mRNA/ protein expression does not distinguish assembled proteasomes from dissociated proteasomes.

The increase in proteasome mRNA following irradiation might logically be thought of as a result of proteasome inhibition caused by any mechanism. To determine if this was the case, we used a proteasome inhibitor, NPI0052, that induces apoptosis in multiple myeloma cells primarily through caspase 8-mediated signaling pathways (12). We observed cytotoxicity of NPI0052 on both U87 and U87EGFRvIII cells within 24 hours while irradiation did not cause cell death in this time frame. Unlike irradiation, NPI0052 inhibited chymotrypsin-like activity almost completely in both U87 and U87EGFRvIII. It is interesting that both NPI0052 and 2 Gy irradiation, which caused

very different levels of inhibition, induced proteasome mRNA expression to a comparable degree in the U87 cells at 6 hours treatment. In U87EGFRvIII cells, NPI0052, in contrast to irradiation, increased expression of core constitutive components but not immuno-subunits. This perhaps was a reflection of the lack of expression of these subunits in the untreated cells suggesting an inhibitory mechanism may be operating to block their expression. In any event, these data imply that cells treated with two separate therapies share the same mechanism of recovery, at least in part. What is clear is that there is a great deal that we do not know about the regulation of proteasome subunit production but that this system is highly dynamic and plastic and capable of responses that are of physiologic importance.

In summary, we have demonstrated using fluorogenic peptides and a physiological substrate of the 26S proteasome that irradiation inhibits the proteasome activity of U87 but not its radioresistant variant U87EGFRvIII. EGFRvIII expression also seems to alter the molecular composition of the proteasomes, with a loss of immuno-subunits. Radiation-induced proteasome inhibition seems to be followed by an increase in mRNA production, which suggests that transcription might be a mechanism of proteasome recovery after irradiation.

Materials and Methods

Cell lines

Human GBM cell lines were maintained in DMEM (Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Omega Scientific, Tarzana, CA) and

penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA). The human glioblastoma

cell line U87MG (U87) was purchased from American Type Culture Collection

(Rockville, MD). The U87EGFR wild type (U87EGFRwt) and U87EGFRvIII cell lines

were provided kindly by Dr. Paul Mischel, Dept. Pathology at UCLA. These cell lines

had been derived by retroviral transduction of U87 cell line with pLPCX constructs that

contain human EGFRwt or EGFRvIII cDNA, respectively, as described earlier (44).

Proteasome inhibitor

The proteasome inhibitor, NPI0052, was a kind gift from Nereus Pharmaceuticals

(San Diego, CA).

Quantitative reverse transcription-PCR

Total RNA was isolated using TRIZOL Reagent (Invitrogen) and then treated with

DNase 1, amplification grade (Invitrogen). cDNA synthesis was carried out using the

TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA)

according to the protocol of the manufacturer. Quantitative PCR was done in the My iQ

thermal cycler (Bio-Rad, Hercules, CA) using the 2x iQ SYBR Green Supermix (Bio-

Rad). Quantitative PCR for each sample was run in triplicate and each reaction contained

1 µL of cDNA in a total volume of 20 µL. ΔC_t for each gene was determined after

normalization to GAPDH and $\Delta\Delta C_t$ was calculated relative to the designated reference

sample. Gene expression values were then set equal to $2^{-\Delta\Delta C_t}$ as described (Applied

Biosystems). All PCR primers were synthesized by Invitrogen and designed for the

human sequence. The following primer pairs (written 5' to 3') were used at the indicated

final concentration: *GAPDH* (at 600 nmol/L), human *c-myc*

(AGCGACTCTGAGGAGGAACA and CTCTGACCTTTTGCCAGGAG at 300

nmol/L), *Lmp2* (ATGCTGACTCGACAGCCTTT and GCAATAGCGTCTGTGGTGAA

at 300 nmol/L), *Y* (AGGAGAAAGATGGCGGCTAC and

AGGACCCAGTGGTTGTTCTG at 300 nmol/L), *Mecl1*

(GTGCTAGAAGACCGGTTCCA and CTCTGTGGGTGAGCTCAGTG at 300

nmol/L), *Z* (GGAGGAGGAAGCCAAGAATC and CTTCCAGCACCTCAATCTCC at

300 nmol/L), *Lmp7* (ATGTCAGTGACCTGCTGCAC and

CTGCTGAGCCCGTACTCTCT at 300 nmol/L), *X* (ACGTGGACAGTGAAGGGAAC

and CTGCATCCACCCTCTTTCAG at 300 nmol/L), *11S PA28 alpha*

(AGACAAAGGTCCTCCCTGTG and CTGGACAGCCACTCCAAAAT at 300

nmol/L), *11S PA28 beta* (ACTCCCTCAATGTGGCTGAC and

GCAGGGACAGGACTTTCTCA at 300 nmol/L), *19S ATPase PSMC1*

(GACCCCGATGTCAGTAGGAA and GGATCCGTGTCATCCATCAG at 300

nmol/L), *19S non-ATPase PSMD4* (CAACGTGGGCCTTATCACAC and

ATTGTCCTCCACTGGGCTTC at 300 nmol/L). The specificity of primers was

validated using melting curve and on the gel.

Western Blot Analysis

Whole-cell lysates were prepared using M-PER mammalian protein extraction reagent

(Pierce, Rockford, IL) with protease inhibitor cocktails (EMD Biosciences, San Diego,

CA). Equal amounts of protein were run on a 10% SDS-PAGE gel. Immunoblotting was

performed with an anti-human 20S proteasome subunit β5 polyclonal AB (Biomol

International, L.P., Plymouth Meeting, PA). An anti-α-Tubulin mouse mAB (EMD

Biosciences) was used as a loading control. Antimouse and rabbit horseradish

peroxidase–conjugated secondary antibodies were from GE Healthcare UK limited

(Buckinghamshire, UK). Immunoblots were developed using SuperSignal West Dura

Extended Duration Substrate (Pierce).

Irradiation

Exponentially growing cells were irradiated using a ^{137}Cs laboratory irradiator

(Mark1, JLShephard) at a dose rate of 5.0 Gy/min.

Clonogenic survival assay

Exponentially growing cells were trypsinized, counted, and diluted accordingly.

These cell suspensions were then irradiated at 2, 4, 6, 8 Gy and plated in 100 mm dishes

in triplicate. After 14 days, colonies were fixed and stained with Crystal Violet in 50%

EtOH. Colonies consisting of more than 50 cells were counted to determine surviving

fractions.

Fluorogenic assay

Chymotrypsin-like proteasome activity was measured using fluorogenic peptide

substrate, Suc-LLVY-AMC (Sigma-Aldrich, St. Louis, MO). 10 μg of cell extracts in

Buffer I (50 mM Tris, 5 mM $MgCl_2$, 2 mM DTT, 2 mM ATP) combined with the

substrate at 100 μM was added into 96-well black plate (Corning Inc., New York, NY)

and the fluorescence of the released AMC was measured for 30 minutes at 37°C using a Tecan SPECTRAFluor Plus fluorometer (TECAN, Durham, NC).

Generation of stable cell lines expressing ZsGreenODC fusion protein using retroviral transduction

The degron from the carboxy-terminal 37 amino acids of ornithine decarboxylase fused to ZsGreen (cODCZsGreen) was digested with BglII and Not1 from pZsProsensor-1 (BD Biosciences) and cloned into the BamHI and EcoRI sites of the retroviral vector pQCXIN (BD Biosciences) using the Not1-EcoR1 DNA oligonucleotide adaptor (New Orleans, LA, EZ Clone Systems). pQCXIN/cODCZsGreen was transfected into GP2-293 pantropic retroviral packaging cells (BD Biosciences) and the collected retrovirus was used to infect U87 and U87EGFRvIII cell lines. Infected cells were then selected under 800 µg/ml of G418 (Invitrogen): U87-cODCZsGreen and U87EGFRvIII-cODCZsGreen. To select transduced cells, single cell clones of U87-cODCZsGreen or U87EGFRvIII-cODCZsGreen cells were isolated by plating dilute suspensions and selecting clones using cloning cylinders (Fisher Scientific, Pittsburgh, PA).

FACS Analysis

Clones were screened for cODCZsGreen fluorescence by FACS analysis. 12 clones of U87-cODCZsGreen and 16 clones of U87EGFRvIII-cODCZsGreen cell lines were irradiated at 10Gy and the fluorescence was measured 6 hour later. Approximately 10^6 cells per each clone were resuspended in 2 ml isotone and analyzed on a FACSCalibur (BD Biosciences).

Confocal Microscopy

Approximately 10^6 cells were plated in 35 mm glass bottom culture dishes (MatTek Corporation, Ashland, MA) overnight. Green fluorescence was detected using a TCS SP MP Inverted Confocal Microscope (Leica, Wetzlar, Germany) after cells were irradiated with 2 Gy.

Statistical Analysis

For quantification of fluorescence due to radiation-induced proteasome inhibition, the flow cytometry data of each clone was analyzed using the Wilcoxon Matched-Pairs Signed-Ranks Test. In order to determine *P* values, the means of fluorescence for each clone with or without radiation treatment were paired.

References

1. Harris JL, Alper PB, Li J, Rechsteiner M, Backes BJ. Substrate specificity of the human proteasome. Chemistry & biology 2001;8(12):1131-41.

2. Li J, Rechsteiner M. Molecular dissection of the 11S REG (PA28) proteasome activators. Biochimie 2001;83(3-4):373-83.

3. Adams J. The proteasome: structure, function, and role in the cell. Cancer treatment reviews 2003;29 Suppl 1:3-9.

4. Coux O. The 26S proteasome. Progress in molecular and subcellular biology 2002;29:85-107.

5. Coux O, Tanaka K, Goldberg AL. Structure and functions of the 20S and 26S proteasomes. Annual review of biochemistry 1996;65:801-47.

6. Akiyama K, Kagawa S, Tamura T*, et al.* Replacement of proteasome subunits X and Y by LMP7 and LMP2 induced by interferon-gamma for acquirement of the functional diversity responsible for antigen processing. FEBS letters 1994;343(1):85-8.

7. Gaczynska M, Rock KL, Spies T, Goldberg AL. Peptidase activities of proteasomes are differentially regulated by the major histocompatibility complex-encoded genes for LMP2 and LMP7. Proceedings of the National Academy of Sciences of the United States of America 1994;91(20):9213-7.

8. McBride WH, Iwamoto KS, Syljuasen R, Pervan M, Pajonk F. The role of the ubiquitin/proteasome system in cellular responses to radiation. Oncogene 2003;22(37):5755-73.

9. Chiarle R, Budel LM, Skolnik J*, et al.* Increased proteasome degradation of cyclin-dependent kinase inhibitor p27 is associated with a decreased overall survival in mantle cell lymphoma. Blood 2000;95(2):619-26.

10. Loda M, Cukor B, Tam SW*, et al.* Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. Nature medicine 1997;3(2):231-4.

11.   Zhang F, Laiho M. On and off: proteasome and TGF-beta signaling. Experimental cell research 2003;291(2):275-81.

12.   Chauhan D, Catley L, Li G, *et al.* A novel orally active proteasome inhibitor induces apoptosis in multiple myeloma cells with mechanisms distinct from Bortezomib. Cancer cell 2005;8(5):407-19.

13.   Richardson PG, Hideshima T, Anderson KC. Bortezomib (PS-341): a novel, first-in-class proteasome inhibitor for the treatment of multiple myeloma and other cancers. Cancer Control 2003;10(5):361-9.

14.   Goel A, Dispenzieri A, Geyer SM, Greiner S, Peng KW, Russell SJ. Synergistic activity of the proteasome inhibitor PS-341 with non-myeloablative 153-Sm-EDTMP skeletally targeted radiotherapy in an orthotopic model of multiple myeloma. Blood 2006;107(10):4063-70.

15.   Goel A, Dispenzieri A, Greipp PR, Witzig TE, Mesa RA, Russell SJ. PS-341-mediated selective targeting of multiple myeloma cells by synergistic increase in ionizing radiation-induced apoptosis. Experimental hematology 2005;33(7):784-95.

16.   Pervan M, Pajonk F, Sun JR, Withers HR, McBride WH. Molecular pathways that modify tumor radiation response. Am J Clin Oncol 2001;24(5):481-5.

17.   Russo SM, Tepper JE, Baldwin AS, Jr.*, et al.* Enhancement of radiosensitivity by proteasome inhibition: implications for a role of NF-kappaB. International journal of radiation oncology, biology, physics 2001;50(1):183-93.

18.   Weber CN, Cerniglia GJ, Maity A, Gupta AK. Bortezomib Sensitizes Human Head and Neck Carcinoma Cells SQ20B to Radiation. Cancer biology & therapy 2007;6(2):156-9.

19.   Pajonk F, McBride WH. Ionizing radiation affects 26s proteasome function and associated molecular responses, even at low doses. Radiother Oncol 2001;59(2):203-12.

20.   Pervan M, Iwamoto KS, McBride WH. Proteasome structures affected by ionizing radiation. Mol Cancer Res 2005;3(7):381-90.

21.   Mischel PS, Cloughesy TF. Targeted molecular therapy of GBM. Brain pathology 2003;13(1):52-61.

22.   Kleihues P, Sobin LH. World Health Organization classification of tumors. Cancer 2000;88(12):2887.

23.   Heimberger AB, Hlatky R, Suki D, *et al.* Prognostic effect of epidermal growth factor receptor and EGFRvIII in glioblastoma multiforme patients. Clin Cancer Res 2005;11(4):1462-6.

24.   Pelloski CE, Ballman KV, Furth AF, *et al.* Epidermal growth factor receptor variant III status defines clinically distinct subtypes of glioblastoma. J Clin Oncol 2007;25(16):2288-94.

25.   Lammering G, Hewit TH, Holmes M, *et al.* Inhibition of the type III epidermal growth factor receptor variant mutant receptor by dominant-negative EGFR-CD533 enhances malignant glioma cell radiosensitivity. Clin Cancer Res 2004;10(19):6732-43.

26.   Lammering G, Hewit TH, Valerie K, *et al.* EGFRvIII-mediated radioresistance through a strong cytoprotective response. Oncogene 2003;22(36):5545-53.

27.   Lal A, Glazer CA, Martinson HM, *et al.* Mutant epidermal growth factor receptor up-regulates molecular effectors of tumor invasion. Cancer research 2002;62(12):3335-9.

28.   Ghoda L, Sidney D, Macrae M, Coffino P. Structural elements of ornithine decarboxylase required for intracellular degradation and polyamine-dependent regulation. Molecular and cellular biology 1992;12(5):2178-85.

29.   Hoyt MA, Coffino P. Ubiquitin-free routes into the proteasome. Cell Mol Life Sci 2004;61(13):1596-600.

30.   Zhang M, Pickart CM, Coffino P. Determinants of proteasome recognition of ornithine decarboxylase, a ubiquitin-independent substrate. The EMBO journal 2003;22(7):1488-96.

31.   Paddison PJ, Silva JM, Conklin DS, *et al.* A resource for large-scale RNA-interference-based screens in mammals. Nature 2004;428(6981):427-31.

32.   James M. Brush KK, Keisuke S. Iwamoto, James W. Sayre, William H. McBride. A reporter gene probe for the effect of irradiation on cellular 26S proteasome function. Submitted.

33.   Lammering G, Valerie K, Lin PS, Hewit TH, Schmidt-Ullrich RK. Radiation-induced activation of a common variant of EGFR confers enhanced radioresistance. Radiother Oncol 2004;72(3):267-73.

34.   McBride WH, Pajonk F, Chiang CS, Sun JR. NF-kappa B, cytokines, proteasomes, and low-dose radiation exposure. Military medicine 2002;167(2 Suppl):66-7.

35.   Pajonk F, McBride WH. The proteasome in cancer biology and treatment. Radiation research 2001;156(5 Pt 1):447-59.

36.   Glickman MH, Raveh D. Proteasome plasticity. FEBS letters 2005;579(15):3214-23.

37.   Bose S, Brooks P, Mason GG, Rivett AJ. gamma-Interferon decreases the level of 26 S proteasomes and changes the pattern of phosphorylation. The Biochemical journal 2001;353(Pt 2):291-7.

38.   Bose S, Stratford FL, Broadfoot KI, Mason GG, Rivett AJ. Phosphorylation of 20S proteasome alpha subunit C8 (alpha7) stabilizes the 26S proteasome and plays a role in the regulation of proteasome complexes by gamma-interferon. The Biochemical journal 2004;378(Pt 1):177-84.

39.   Satoh K, Sasajima H, Nyoumura KI, Yokosawa H, Sawada H. Assembly of the 26S proteasome is regulated by phosphorylation of the p45/Rpt6 ATPase subunit. Biochemistry 2001;40(2):314-9.

40.   Mason GG, Murray RZ, Pappin D, Rivett AJ. Phosphorylation of ATPase subunits of the 26S proteasome. FEBS letters 1998;430(3):269-74.

41.   Contessa JN, Hampton J, Lammering G*, et al.* Ionizing radiation activates Erb-B receptor dependent Akt and p70 S6 kinase signaling in carcinoma cells. Oncogene 2002;21(25):4032-41.

42.   Snyder AR, Morgan WF. Radiation-induced chromosomal instability and gene expression profiling: searching for clues to initiation and perpetuation. Mutat Res 2004;568(1):89-96.

43.   Szkanderova S, Vavrova J, Hernychova L, Neubauerova V, Lenco J, Stulik J. Proteome alterations in gamma-irradiated human T-lymphocyte leukemia cells. Radiation research 2005;163(3):307-15.

44.   Wang MY, Lu KV, Zhu S*, et al.* Mammalian Target of Rapamycin Inhibition Promotes Response to Epidermal Growth Factor Receptor Kinase Inhibitors in PTEN-Deficient and PTEN-Intact Glioblastoma Cells. Cancer research 2006;66(16):7864-9.

Acknowledgements

Figure Legends

FIGURE 1. Effect of ionizing radiation on U87 and U87EGFRvIII cell lines. **A.** Clonogenic assay comparing U87EGFRvIII to the parental line U87. Cells were trypsinized, irradiated with 2, 4, 6, or 8 Gy, and then plated in 100 mm dishes in triplicates. After 14 days, the colonies were fixed and stained with Crystal Violet. The colonies containing more than 50 cells were counted. Radiological parameters obtained from a linear-quadratic fit: U87 α=0.175, β=0.031, α/β=5.57; U87EGFRvIII α=0.272, β=0.004, α/β=66.4. **B.** Proteasome chymotrypsin-like activity was measured by fluorogenic assay using Suc-LLVY-AMC peptide following 10 Gy irradiation. The average fluorescent value in quadruplicate from a representative experiment is shown. Asterisks note significant differences between 0 Gy and 10 Gy for each cell line calculated by Student's t test; ($P <$ 0.001).

FIGURE 2. Determination of cODCZsGreen fluorescence of U87-cODCZsGreen and U87EGFRvIII-cODCZsGreen stable lines after radiation. **A.** The accumulation of proteasome substrate, cODC, following compromised proteasome function was measured by the expression of cODCZsGreen in unirradiated and irradiated clones using flow cytometry. 12 clones of U87-cODCZsGreen and 16 clones of U87EGFRvIII-cODCZsGreen cells were irradiated at 10 Gy and their fluorescence was assessed 6 hours after irradiation. Each bar displays the mean fluorescence of individual irradiated clone measured by flow cytometry after normalization to that of its untreated control and plotted as a percentile (the values above 100% reflect the increase above untreated).

The inhibition of proteasome activity following radiation, which resulted in the increased fluorescence, was statistically significant in U87-cODCZsGreen (P <0.005, calculated by Wilcoxon Matched-pairs Signed-Ranks Test) while it was not statistically significant in U87EGFRvIII-cODCZsGreen (P =0.298). **B.** Three clones of U87-cODCZsGreen were further analyzed by confocal microscopy and a representative image following irradiation (marked as * in A) is shown. Bar = 50 μM. The inhibition of proteasome function was detected in U87 as early as 15 minutes and prominent at 45 minutes after 2 Gy irradiation.

FIGURE 3. qRT-PCR analysis of proteasome subunits from U87, U87EGFR, and U87EGFRvIII. Cells in logarithmic growth were used for mRNA expression analysis of 10 different proteasome subunits. PCR reactions were quantified by SyberGreen in the My iQ thermal cycler (Bio Rad). The expression of each gene was normalized to a house keeping gene, GAPDH and compared to a constitutive subunit X (where the X value was set equal to 1).

FIGURE 4. mRNA and protein analysis of proteasome subunits in U87 and U87EGFRvIII cell lines after ionizing radiation. **A.** qRT-PCR analysis at 6 hours after 2Gy radiation. **B.** qRT-PCR analysis at 24 hours after 2Gy radiation. The expression of each gene was normalized to GAPDH and then compared to 0 Gy control samples (0 Gy equals 1). An increase in expression was observed in all of the proteasome subunits of U87 cell line at 6 hours while an increase was no longer observed at 24 hours after irradiation. **C**. Immunoblot analysis at 6 hours

after irradiation with 2 Gy or 10 Gy. Immunoblotting was performed with an anti-human 20S proteasome subunit β5 polyclonal antibody. An anti-α-tubulin antibody was used as a loading control. Each band was quantified using Image J (NIH), normalized to α–tubulin and then compared to 0 Gy sample (0 Gy equals 1).

FIGURE 5. Gene expression of proteasome subunits after proteasome inhibitor treatment. **A**. Cells were treated with 50 nM NPI0052 or vehicle (0.25% DMSO) for 3 hours then washed, and RNA collected 6 hours post-treatment. The expression of each gene was normalized to GAPDH and 0 nM NPI0052. Unlike radiation, NPI0052 induced the mRNA expression of constitutive-subunits in U87EGFRvIII. **B**. A summary diagram of mRNA expression changes in proteasome subunits. Fold changes of constitutive-subunits (X, Y, Z) and immuno-subunits are shown after ionizing radiation or a proteasome inhibitor NPI0052 treatment (U87; filled boxes: U87EGFRvIII;open boxes). The constitutive-subunits were induced by NPI0052 but not by irradiation in U87EGFRvIII.
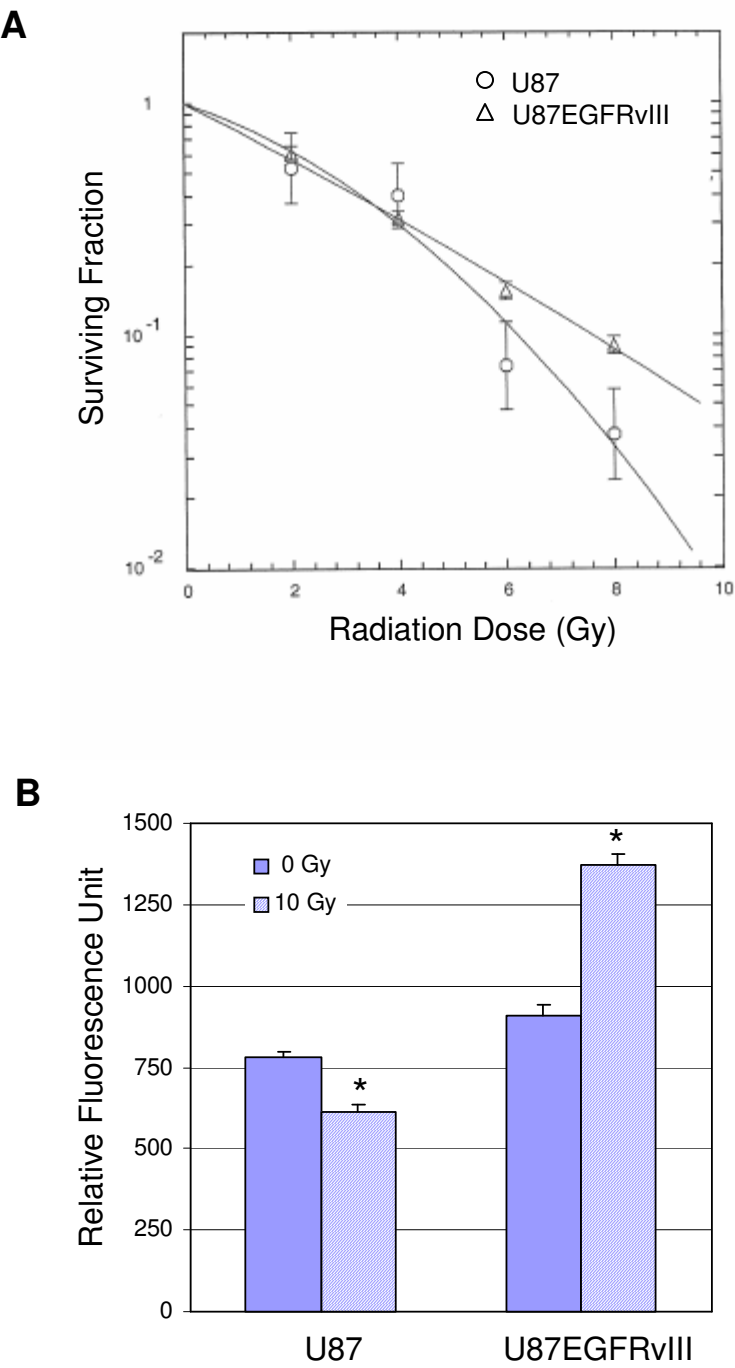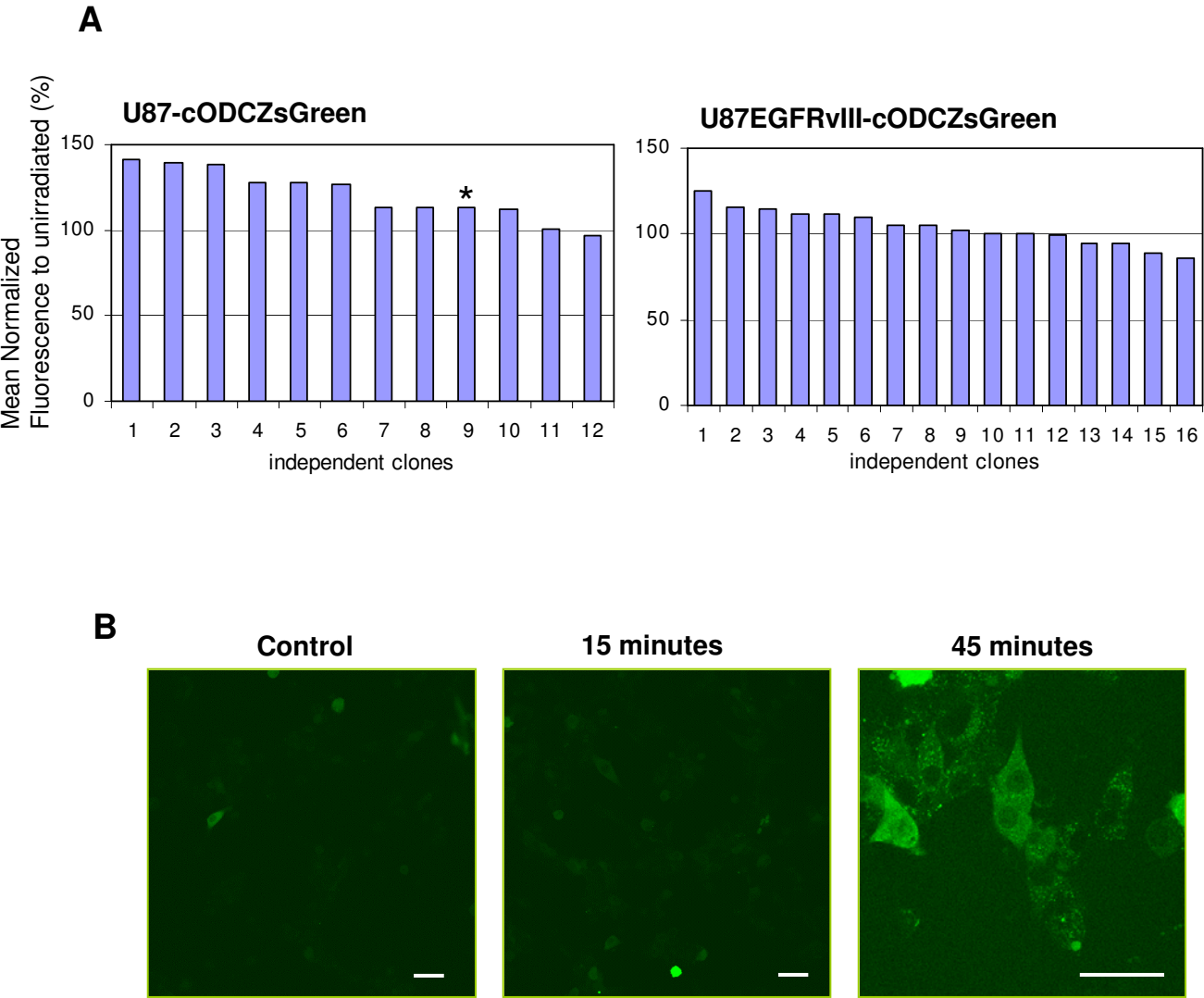
Figure 1

A

B
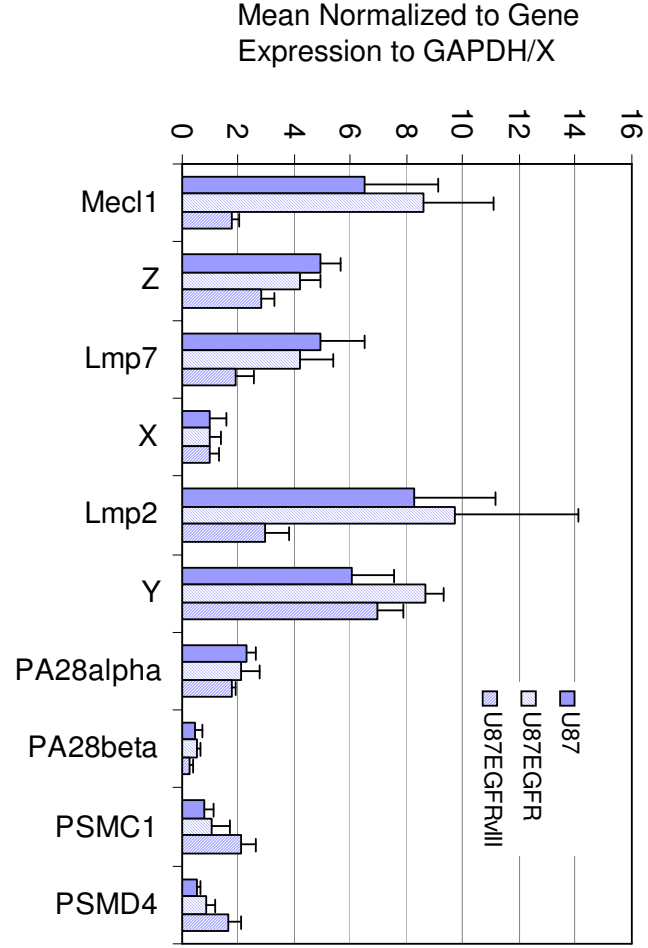
Figure 2

A

B

Figure 3

Figure 4

Figure 5

A

50 nM NPI0052

Y-axis: Mean Normalized Gene Expression to GAPDH/ vehicle control

Legend:
- U87
- U87EGFRvIII

X-axis categories: Mecl1, Z, Lmp7, X, Lmp2, Y, PA28alpha, PA28beta, PSMC1, PSMD4

B

Categories:
- U87 constitutive
- U87 immuno
- U87EGFRvIII constitutive
- U87EGFRvIII immuno

Left chart X-axis: Fold changes after IR

Right chart X-axis: Fold changes after NPI0052